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Identification of Neopterin as a Potential Indicator of Infection in Burned Patients

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Abstract. Several fluorescent substances are present in the supernatants of acid-precipitated whole blood or plasma from burned patients. Perchloric acid supernatants of sera from infected, but not uninfected, burned rats contained a fluorescent substance with maximum emission at 420 nm at 355-nm excitation (355 ex/420 em). In this study of serum from burned human patients, several fluorescent substances were resolved by reverse-phase high-pressure liquid chromatography. One of these fluorescent components had an high-pressure liquid chromatography retention time and fluorescent characteristics identical to those of neopterin. The identification of this component as neopterin was verified by thermospray mass spectrometry. Serum neopterin concentrations were then determined in supernatants of patient serum samples having various levels of 355 ex/420 em fluorescence. A correlation was found between the concentrations of neopterin determined by high-pressure liquid chromatography and the presence of bacteremia in burned patients. These findings suggest that neopterin, which is a useful indicator of infection in other clinical settings, may also be an indicator of infection in burned patients.

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Infection poses a serious threat to all severely burned patients and is a persistent obstacle to successful therapy. Prompt diagnosis of infection is crucial for timely treatment and for patient survival. Systemic changes induced by burn injury, such as leukocytosis and fever, hamper the early detection of systemic infection and make its diagnosis difficult in such patients. Rapid biochemical detection of the presence of infection, if reliable, could provide diagnostic verification more promptly than is currently possible with standard microbiologic techniques.

Abnormal levels of hormones (1, 2), acute phase proteins (3, 4), and fluorescent substances (5) in blood and plasma occur in the presence of inflammation or infection in burned humans and animals. Their presence in blood and plasma may reflect a metabolic response to both trauma and infection. Although bio-

chemical indices specific to the invading species are desirable, the spectrum of microbes to be differentiated and their low levels in the blood early in infection limit the likelihood of developing such indices. As an alternative, a biochemical change in serum or blood specific to infection itself would permit early identification of this state, and might have diagnostic utility.

We have purified and biochemically characterized substances from the blood of burned patients in which 420-nm fluorescence emission at 355-nm excitation (355 ex/420 em) was detected in perchloric acid (PCA) supernatants. These substances offer some promise as specific indicators of the presence of infection.

Materials and Methods

Fluorescence Measurement of PCA Supernatants. To measure fluorescent indicators in PCA supernatants, 1 ml of anticoagulated blood or plasma was mixed with 4 ml of cold (4°C) PCA (0.8 M). After incubating for 10 min at 4°C, the mixture was centrifuged at 4°C for 10 min at 3,000g; the supernatant was centrifuged again at 20,000g for 30 min. This clear supernatant was then transferred and fluorescence was measured using an Aminco Bowman (SLM Instruments, Inc., Urbana, IL) spectrofluorometer at 355-nm excitation and 420-nm emission (355 ex/420 em). The

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fluorometer was standardized using a calibration standard fluorescence intensity block.

Determination of Serum Pterin Levels. Pterin levels were determined in 200- μ l samples of serum that were deproteinized by adding 400 μ l of 0.2 M potassium phosphate buffer (pH 4.5) and incubating at 100°C in an oil bath for 20 min. This mixture was centrifuged at 20,000g for 20 min and 100 μ l of the supernatant was injected directly on a Hewlett Packard (Palo Alto, CA) model 1090 liquid chromatograph with a Biophase ODS reverse phase 4.6 \times 250-mm column (Bioanalytical Systems, West Lafayette, IN). The mobile phase consisted of 0.05 M ammonium acetate (pH 7.0) for 7 min followed by a methanol gradient reaching 30% in 4 min. Column temperature was maintained at 45°C and the flow rate was 1.0 ml/min. The high-pressure liquid chromatography (HPLC) was equipped with a Kratos (Kratos Analytical, Ramsey, NJ) fluorescence detector, model 980, with a 25- μ l flow cell. The excitation monochromator was set at 350 nm and the emission cutoff filter was at 389 nm. The retention times for standard pterins (Sigma Chemical Co., St. Louis, MO) were determined using 100- μ l aliquots of a standard solution of pterins (10 ng/ml). The amount of each fluorescent substance present was determined using a Hewlett Packard model 3392A integrator. Various amounts of standard neopterin were analyzed and the integration results were used to construct a standard curve for the determination of unknown levels of neopterin.

Identification of Neopterin by HPLC Mass Spectrometry. Serum supernatants were purified by ion exchange chromatography after heat denaturation and separation of the serum proteins by a modification of the method of Stea *et al.* (6). The supernatants were passed through a cation exchange membrane (Bio-Rad Laboratories, Richmond, CA), washed with distilled water, and eluted directly onto an anion exchange membrane (Bio-Rad) with 3 ml of 0.1 N NH_4OH . The anion exchange membrane was washed with distilled water and the sample was eluted with 3 ml of 1 N formic acid. The ion exchange eluate was concentrated under a stream of nitrogen. The sample was further purified by multiple 20- μ l injections of the concentrated sample and collection of the column eluent corresponding to the neopterin retention time. The pooled fractions were concentrated to 100 μ l under a stream of nitrogen. A portion (20 μ l) of the concentrate was injected into the HPLC and the column effluent was directed to the thermospray module of the mass spectrometer. Mass spectral analysis was performed on a Hewlett Packard model 5988 TS-LC/MS with a thermospray LC-MS interface. The HPLC column was a C-18 reverse phase, the flow rate was 1 ml/min, and the elution buffer was 0.1 M ammonium acetate buffer.

Patient Data. Remnants of sera collected from 24

patients for clinical analysis were used to examine the neopterin levels during infection. The burns in these patients averaged 47.3% of the total body surface and ranged from 19% to 72%. The patient ages averaged 34.9 years and ranged from 20 to 66 years. Clinical diagnoses of infection were based on standardized criteria utilizing both clinical and laboratory data (7). Computer records were retrospectively screened to determine the types of infection experienced by each patient.

Data Analysis. Statistical analysis was performed using BMDP statistical software (BMDP Statistical Software, Inc., Los Angeles, CA). Groups were compared by a pairwise *t* test with a Bonferroni adjustment using program P7D.

Results

The optimal chromatographic conditions described in Materials and Methods were determined for the chromatography of the 355 ex/420 em factors. The chromatograph obtained for a mixture of pterins under these conditions is shown in Figure 1. Neopterin had a retention time of 5.4 min, isoxanthopterin 8.3 min, and biopterin 9.4 min. A patient serum sample that had a relatively high fluorescence at 355 ex/420 em after perchloric acid precipitation was deproteinized by heat and analyzed by HPLC. The chromatogram so obtained is compared with a sample extracted from a normal unburned control in Figure 2. Three of the peaks in the patient sample had retention times similar to neopterin, isoxanthopterin, and biopterin, respectively (Figure 1), while none of the same peaks were present in the control sample.

Since the fluorescence characteristics and HPLC of one of the substances were similar to those of neopterin, mass spectral analysis was performed on the partially purified sample in order to verify the identity of the peak corresponding to neopterin. Because the sensitivity of the mass spectrometer was less than that of the fluorescence detector, a pool of serum samples (3 ml)

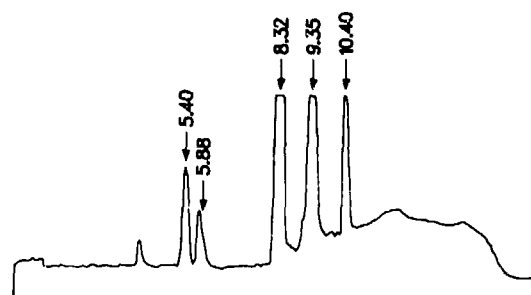


Figure 1. Chromatography of neopterin and five other pterins. A μ l aliquot of a standard pterin solution was chromatographed under the conditions described. This solution contained 10 μ g/ml of pterin-6-carboxylic acid (4.0 min), neopterin (5.4 min), xanthopterin (5.88 min), isoxanthopterin (8.32), biopterin (9.35 min), and 6-methyl pterin (10.4 min).

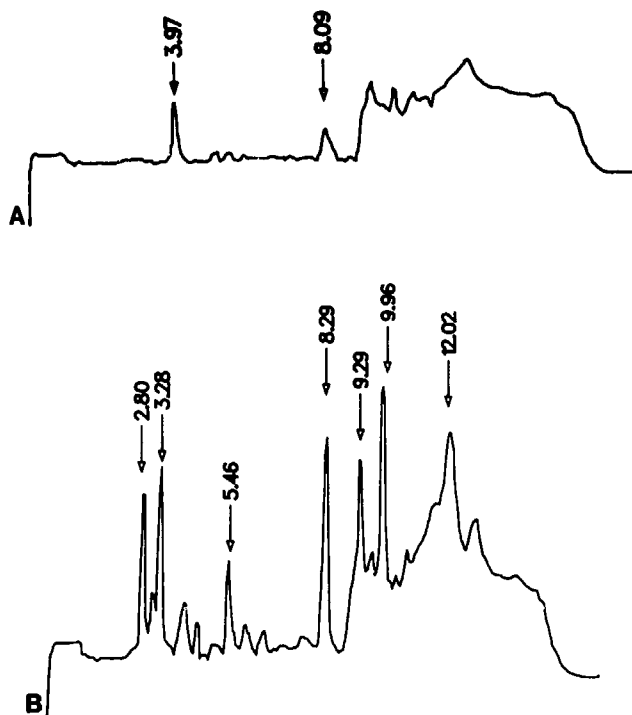


Figure 2. Comparison of chromatograms from a highly fluorescent patient sample and a normal control. Chromatography conditions were as described in Materials and Methods. Relevant peaks are labeled. (A) Control sample. (B) Patient sample.

from several patients with high 355 ex/420 nm fluorescence was deproteinized and the supernatant was purified by ion exchange chromatography. The eluate from the ion exchange column was concentrated and repeatedly chromatographed by HPLC. The eluate of the peak corresponding to neopterin was collected after each injection. The collections were pooled and concentrated by evaporation. Confirmation of the identity of the unknown peak as neopterin was obtained by comparison of the unknown peak with that of a known neopterin standard. Since the fluorescence detector and the mass spectrometer could not be run simultaneously, the column eluate was diverted to the mass spectrometer and compounds separated on the HPLC column were detected by monitoring the neopterin parent ion (254 amu). Specific monitoring for the parent ion of neopterin ($m + 1$, 254 amu) revealed the presence of neopterin (Fig. 3) in the HPLC-purified sample.

A group of burned patient's sera with increased levels of PCA-precipitated supernatant fluorescence was selected for analysis. Neopterin concentration was measured in each of the sera. A representative sample of the chromatograms obtained from HPLC is shown in Figure 4. All of the patients shown in Figure 4 experienced infection during their hospital course. Samples from four (Patients b, c, d, and f, Fig. 4) of the five patients were taken less than 11 days after a clinically defined infection. Neopterin had a retention time of

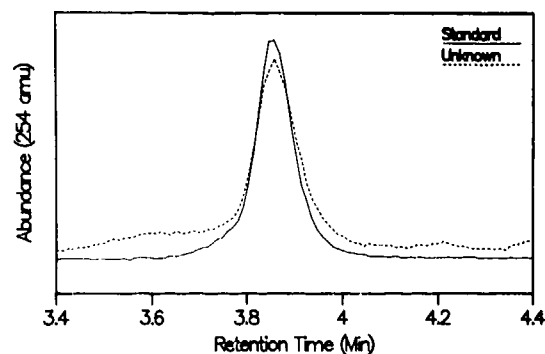


Figure 3. Comparison of high-pressure liquid chromatograms of partially purified patient sera and standard neopterin using mass spectral single ion monitoring for detection. Effluent from HPLC separation of the ion exchange purified pool of patient sera was directed into the thermospray module of a mass spectrometer. The parent ion ($M + 1 = 254$ amu) for standard neopterin was monitored specifically to detect neopterin.

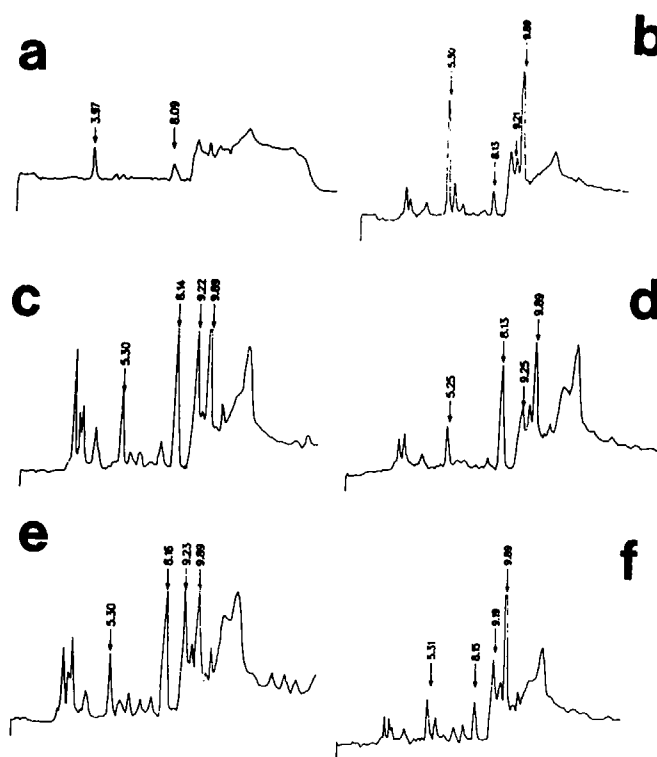


Figure 4. Comparison of high-pressure liquid chromatograms of patient samples with high fluorescence readings with an unburned control sample. Five patient samples with high values for the 355 ex/420 nm factor were chromatographed by HPLC with a fluorescence detector. Peaks were most consistently present at 5.3 min, 8.1 min, 9.2 min, and 9.9 min in the patient samples. (a) Unburned control; (b) through (f) patient samples.

5.3 min under the conditions employed when these chromatograms were obtained. All five patients had levels of neopterin above that found in the control. In addition to neopterin, three other fluorescent substances were present consistently in the PCA supernatants. These fluorescent substances had retention times

of approximately 8.1, 9.2, and 9.9 min. The 8.1-min and 9.2-min retention times corresponded to the retention times for standard solutions of isoxanthopterin and biopterin respectively. Further studies are being conducted to identify these substances. Combined, the neopterin and the other three recurring peaks account for an average of 72.1% of the fluorescence measured during the first 10 min of analysis by HPLC under these conditions.

Neopterin concentrations in unburned controls were relatively low. We analyzed serum samples from 22 healthy individuals for neopterin and determined a mean value of $3.177 \text{ ng/ml} \pm 0.45$. This value compares to 1.35 ng/ml determined by Werner *et al.* (8) and Haas and Gerstner (9) from blood bank samples using radioimmunoassay, and 5.52 ng/ml determined by Słazzyk and Spierto (10) using HPLC.

As a preliminary evaluation of the usefulness of neopterin in diagnosing infection, the neopterin values for the group of patients were matched with the clinical microbiology information available for these patients. Since neopterin is cleared from the circulation rather rapidly, increases due to the presence of infection can only be reliably determined in patients with adequate renal function. Serum creatinine values were used to exclude sera that might have come from patients with renal insufficiency. Only serum neopterin values from sera with creatine levels below 2 mg/dl were included in the analysis.

Two hundred seventy-one serum samples were selected for this study. Of these, 30 samples had a serum creatinine concentration of 2 mg/dl or more and were removed from further analysis. The remaining 241 samples were from 25 patients. Of the 25 patients, all but seven had experienced at least one infectious episode during the period when samples were collected. Most patients had more than one infection. The number and types of these infections are shown in Table I.

The mean neopterin concentrations in patients who had experienced various types of infections are shown in Figure 5. The mean concentration for all the patient samples was $14.33 \pm 0.81 \text{ ng/ml}$ or $16.12 \pm$

0.90 ng/mg/dl creatinine ($n = 241$). Samples from patients who experienced episodes of bacteremia had the highest levels of serum neopterin, compared with samples from patients with other infections or no infection ($P < 0.0001$). The results were further analyzed with respect to the time of diagnosis of infection. As shown in Figure 6, mean neopterin concentrations from all patients with infections were highest in the periods from 10 days before infection to 19 days after infection, compared with samples taken from other periods of time.

A temporal depiction of the course of serum neopterin concentrations for the four bacteremic patients is shown in Figure 7. Serum neopterin was elevated as early as 9 days before the detection of bacteremia and gradually declined after the sixth day after diagnosis. Similar plots of the other infection groups failed to show any sustained increases in neopterin concentration above that of the mean for the uninfected group.

Discussion

We have consistently found four fluorescent components in the sera of burned patients. These substances have fluorescence characteristics similar to nucleotide derivatives such as the pterins, and three of the components copurify with and are chromatographically similar to pterin standards. We have established the chemical identity of one of these substances as neopterin.

Neopterin is secreted by activated cultures of mononuclear leukocytes, specifically from macrophages stimulated by γ -interferon (11). Neopterin is excreted in increased amounts in humans whose immune systems are responding to viral infection, (12) including human immunodeficiency virus (13, 14), or tuberculosis (15), and has been used both as a prognostic indicator for certain kinds of cancer (16) and as an index of transplant rejection (17). Despite its widespread presence in the body, no physiological role for neopterin has been found. Its precursor, dihydroneopterin triphosphate, is also a precursor of tetrahydrobiopterin, which, in turn, is a cofactor in hydroxylation reactions, particularly the hydroxylation of phenylalanine to form tyrosine, the precursor of serotonin and the catecholamines.

This preliminary evaluation suggests that increased plasma neopterin may be an indicator of bacteremia in burned patients. In the present group of patients, neopterin concentrations were consistently elevated for a few days before and after the detection of bacteremia. However, this study included only four patients with bacteremia and seven without any infection at all, and since neopterin concentrations appear to be moderately elevated in burned patients without evidence of infection, the study does not permit explicit attribution of the observed further increases in infected patients to

Table I. Number and Type of Infections

Type	Occurrences (n)	Patients (n)
Cellulitis	6	6
Pneumonia	14	11
Wound	3	3
Bacteremia	4	4
Urinary tract	5	4
Miscellaneous*	7	5
No infection	—	7

* Miscellaneous infections included bronchitis, eye infection, subcutaneous abscess, and infection site unknown.

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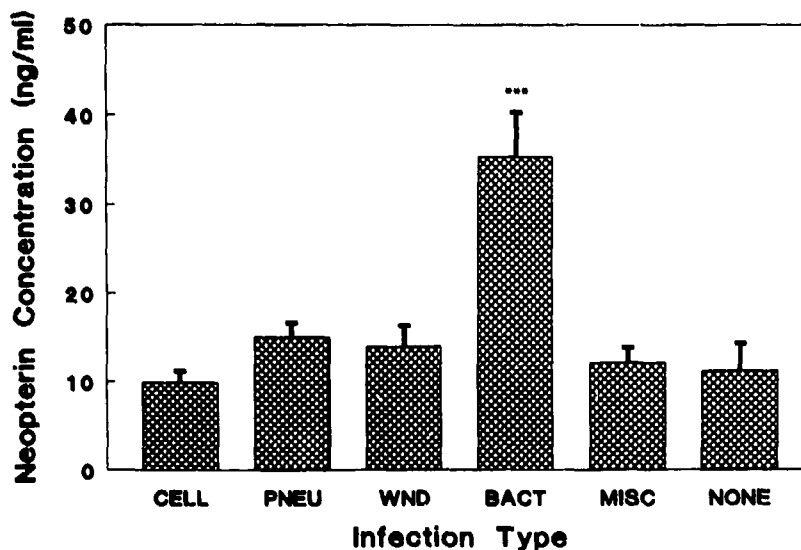


Figure 5. Mean serum neopterin concentration for sera from patients with various types of infections or no infection. The bars represent the mean values taken for all patients with a particular infection of samples taken in between 10 days before and 19 days after infection diagnosis. The upper bar represents the standard error of the mean. *** $P < 0.0001$.

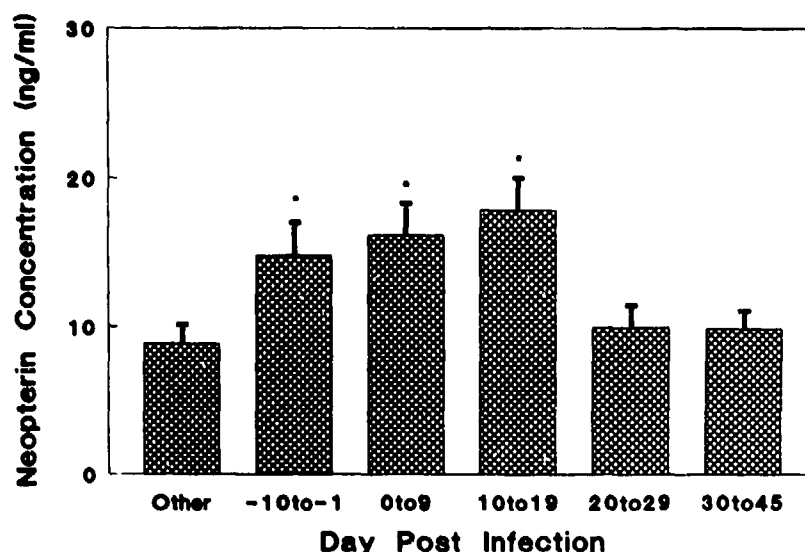


Figure 6. Mean serum neopterin values for various times before and after infection. Values shown on the x axis are the days relative to the day of infection diagnosis and start of treatment. The bars represent the mean neopterin value for all patients with infection who fell within the time window indicated regardless of type of infection. The upper bars represent the standard error of the mean. * $P < 0.05$.

infection alone. A greater number of patients need to be studied to ascertain whether neopterin is clinically useful in signaling the presence of bacteremia or other types of infection after burn injury. A prospective study is underway to collect such data.

In other patients, changes in neopterin levels have been most dramatic in cases of viral and intracellular infection, presumably reflecting the involvement of γ -interferon in the cellular-mediated immune response to these agents. There has been little previous evidence that bacterial infections elicit a change in neopterin excretion, but several recent studies suggest the presence

of increased serum neopterin levels in such infections. Kellermann *et al.* (18) report elevated levels in 27 septic patients; trauma patients with no evidence of bacteremia did not exhibit this increase. These patients were not further stratified with respect to clinical diagnosis. Studies by Strohmaier *et al.* (19), and more recently by Pacher *et al.* (20) from the same laboratory, assessed plasma neopterin levels in patients with acute respiratory distress syndrome (ARDS) and multiorgan failure, respectively. In both studies, it was possible to differentiate septic from nonseptic patients on the basis of serum neopterin concentrations. In the former study,

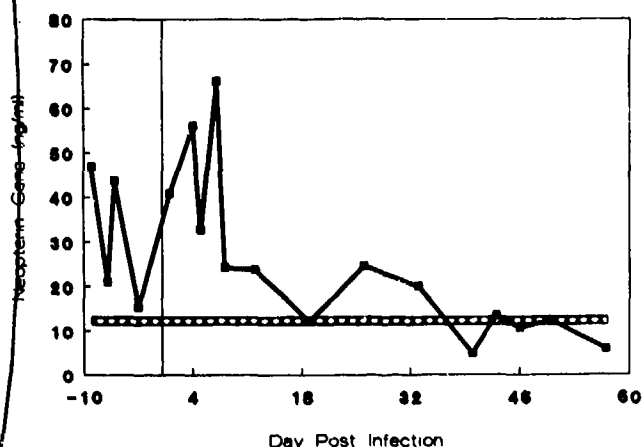


Figure 7. Temporal plot of serum neopterin concentration in bacteremic burn patients. Serum neopterin levels are plotted for four patients who were bacteremic during their course of recovery. Values are plotted with reference to the day that infection was diagnosed. Values occurring on the same post-infection day were averaged. The mean neopterin concentration \pm SE for patients who had no infection is represented by the shaded horizontal bar.

it was also possible to separate surviving septic ARDS patients from nonsurviving ARDS patients on the basis of observed serum neopterin concentrations.

In the burned patients studied here, serum neopterin levels were increased, and increased further with bacteremia. Neopterin is relatively easy to identify and measure in body fluids by HPLC, and a radioimmunoassay has also been developed for its measurement (21, 22). These facts, combined with its known utility as an indicator of infection in other clinical conditions, suggest a possible role for serum neopterin as an indicator of infection in burned patients.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

Human subjects participated in these studies after giving their free and informed voluntary consent. Investigators adhered to AR 70-25 and USAMRDC Reg 70-25 on Use of Volunteers in Research.

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